



REMARKS

TECH CENTER 8600/2000

AUG 30 2001

RECEIVED

The above amendments to the specification and claims have been made to place the application in better condition for examination. Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changes made.**"

In the event that any fees are due in connection with this paper, please charge our Deposit Account No. 01-2340.

Respectfully submitted,

ARMSTRONG, WESTERMAN, HATTORI,  
MCLELAND & NAUGHTON, LLP

William F. Westerman  
Attorney for Applicants  
Reg. No. 29,988

Atty. Docket No. 001554  
1725 K Street, N.W., Suite 1000  
Washington, DC 20006  
Tel: (202) 659-2930  
Fax: (202) 887-0357  
WFW/nrp

Attached: Version with markings to show changes made



RECEIVED  
AUG 30 2001  
TECH CENTER 1600/2940

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Please replace the paragraph beginning at line 23, Page 1 and continuing to Page 2, with the following rewritten paragraph:

Conventionally, two methods have widely been employed for culturing epithelial cell, particularly epidermal cell (or which is called epidermal keratinocyte). One utilizes sterilized 3T3 mouse ~~lung~~ embryo fibroblast, i.e., viable 3T3 mouse lung embryo fibroblast from which division and proliferation potencies have been deleted by irradiating, for example,  $\gamma$  ray or by adding an agent such as mitomycin C, as feeder layer (such as the feeder layer culture method described in James G. Rheinwald and Howard Green. Cell 6: 331-344. Serial Cultivation of Strains of Human Epidermal Keratinocytes: the Formation of Keratinizing Colonies from Single Cells). The other utilizes serum-free medium such as MCDB153 instead of feeder layer.

Please replace the paragraph beginning at line 9, Page 2, with the following rewritten paragraph:

However, the conventional feeder layer culture method in which 3T3 mouse ~~lung~~ embryo fibroblasts are used as a feeder layer involves complicated procedure for preparing the 3T3 mouse ~~lung~~ embryo fibroblasts immediately before epidermal cells are inoculated, and such feeder layer has a limited life time. During proliferation of epidermal cells other than mouse epidermal cells such as human epidermal cells and preparation of an epidermal cell sheet, those cells may possibly be contaminated with heterogenous cells, 3T3 mouse ~~lung~~ embryo fibroblasts, and an agent such as mitomycin C, which is added to delete division and proliferation potencies of the 3T3 mouse ~~lung~~ embryo fibroblasts, may remain.

Please replace the paragraph beginning at line 20, Page 2 and continuing onto Page 3, with the following rewritten paragraph:

On the other hand, when fibroblast homogenous to the epidermal cell and/or epidermal cell sheet of interest is used as the feeder cell instead of 3T3 mouse ~~lung~~ embryo fibroblast (e.g., human fibroblast may be used for preparing a human epidermal cell sheet), there is no possibility of contamination with heterogenous cells. However, their division and proliferation potencies should also be deleted by adding an agent such as mitomycin C that may possibly remain. Human fibroblast may be used, but they provide slower proliferation rate of the epidermal cells when compared to that obtained by using 3T3 mouse ~~lung~~ embryo fibroblast.

Please replace the paragraph beginning at line 3, Page 3, with the following rewritten paragraph:

Culture method employing serum-free medium may often provide slower proliferation rate of epidermal cell and require longer period of time for incubation when compared to feeder layer culture methods which employ feeder cells such as 3T3 mouse ~~lung~~ embryo fibroblast. Further, serum-free medium is likely to suppress the differentiation of epidermal cell, which may cause inability of the epidermal cell to form a multiple-layer, resulting in unsuccessful preparation of an epidermal cell sheet.

Please replace the paragraph beginning at line 6, Page 5, with the following rewritten paragraph:

Still another object of the present invention is to provide a culture vessel which can provide improved adhesion to cell and enhanced cell-proliferation according to the steps of the above method. Particularly, one object of the present invention is to provide a culture vessel which can provide improved adhesion to cell and enhanced cell-proliferation, which are manufactured by culturing and killing fibroblasts derived from a mammal (particularly 3T3 mouse ~~lung~~ embryo

fibroblast) by, for example, freezing and/or drying in a culture vessel and separating the killed fibroblasts from the vessel at least partially to substantially leave a component or components such as the accumulated extracellular matrix which has been secreted from the culture cells to remain on the surface of the culture vessel, i.e., to leave component(s) necessary for cell adhesion and proliferation to remain on the surface of the culture vessel.

Please replace the paragraph beginning at line 8, Page 6, with the following rewritten paragraph:

In the method, 3T3 mouse ~~lung~~ embryo fibroblast may be used as fibroblast derived from a mammal.

Please replace the paragraph beginning at line 25, Page 6, with the following rewritten paragraph:

For the culture vessel, 3T3 mouse ~~lung~~ embryo fibroblast is used as fibroblast derived from a mammal preferably.

Please replace the paragraph beginning at line 11, Page 8, and continuing to Page 9, with the following rewritten paragraph:

Fibroblasts may be used those derived from mammals such as mouse, human, rat, hamster and rabbit. Preferably, 3T3 mouse ~~lung~~ embryo fibroblast, which is commonly used in conventional feeder layer culture methods, may be used. The condition for inoculating and culturing cell is not particularly limited, and any standard condition may be used. For example, fibroblasts grown in a culture vessel may be separated by treating with trypsin solution (which was prepared by dissolving trypsin (0.25 weight/volume %) in a solution of 0.206 mg.ml ethylenediamine-tetraacetic acid (EDTA) in phosphate buffer). The separated fibroblasts were suspended in a medium supplemented with 5 to 10% fetal bovine serum, inoculated in the culture vessel, and then left to stand in a CO<sub>2</sub>

incubator. No special culture vessel, for example, a culture vessel coated with extracellular matrix such as collagen, is required. Any material or shape may be used for the culture vessel as long as 3T3 fibroblasts, for example, can adhere to and proliferate in the culture vessel. Any culture vessel for adhesive cell which are commercially available such as flask, petri dish, roller bottle, well plate or tray, or any carriers such as conventional synthetic polymer membrane, film or plate, or biopolymer membrane, film or microbeads may be used, which can greatly reduce the process costs when compared to any conventional methods.

Please replace the paragraph beginning at line 12, Page 12, with the following rewritten paragraph:

In this process, fibroblasts should be completely removed in order to prevent heterogenous cells such as 3T3 mouse ~~lung~~ embryo fibroblast from contaminating the epidermal cell sheet, epidermal cell suspension or hepatic cells obtained by using the culture vessel manufactured according to the steps of the above cell adhesion and proliferation method, which comprises inoculating, culturing and then killing fibroblast derived from a mammal to provide improved cell adhesion and proliferation. However, complete removal of fibroblasts may not always be necessary in the aspect of cell adhesion and proliferation. Accordingly, the dead cells may be removed at least partially such that cell adhesion or proliferation may not be inhibited. The term “be separated at least partially” herein, which refers to the extent of removal of dead cells, particularly means removing preferably 50% or more, more preferably 80% or more, and most preferably 100% of dead cells when compared to the total amount of viable fibroblasts just before killed.

Please replace the paragraph beginning at line 5, Page 13, with the following rewritten paragraph:

The extent to which dead cells are removed can be easily confirmed by using, for example, a phase contrast microscope. When 100% removal of dead cells is confirmed, then it means that the

resultant epidermal cell sheet, epidermal cell suspension or hepatic cells are free of heterogenous cells such as 3T3 mouse ~~lung~~ embryo fibroblast. It is not difficult to remove 100% of dead cells. Dead cells can be easily removed almost completely by any conventional procedure. Alternatively, dead cells will completely be apart from the surface of the vessel into the culture solution. Therefore, an epidermal cell sheet, an epidermal cell suspension or hepatic cells obtained by using the culture vessel according to the present invention which can provide improved adhesion to cell and enhanced cell-proliferation may be substantially free of any heterogenous cell. Conventionally, a practical epidermal cell sheet could be prepared only by feeder layer culture method which may cause contamination with heterogenous cells to some extent when cells derived from a mammal other than human are used as the feeder cells. On the other hand, an epithelial cell sheet, i.e., a mucosa epithelial cell sheet or a mucosa epithelial cell suspension, or an epidermal cell sheet or an epidermal cell suspension obtained by using the culture vessel according to the present invention which can provide improved adhesion to cell and enhanced cell-proliferation are free of heterogenous cells.

Please replace the paragraph beginning at line 16, Page 15, with the following rewritten paragraph:

Established 3T3 mouse ~~lung~~ embryo fibroblasts were inoculated in a culture flask (culture surface: 25 cm<sup>2</sup>) at  $3 \times 10^3$  cells/cm<sup>2</sup> and incubated in a CO<sub>2</sub> incubator (at 37°C, 5% CO<sub>2</sub>) for 4 days. The medium was Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (DMEM + 10%FBS).

Please replace the paragraph beginning at line 4, Page 16, with the following rewritten paragraph:

On the other hand, epidermal cells were inoculated at  $1 \times 10^4$  cells/cm<sup>2</sup> in a culture flask (culture surface: 25 cm<sup>2</sup>) containing a feeder layer consisting of 3T3 mouse ~~lung~~ embryo fibroblasts

which have been treated with mitomycin C to delete their division potency as a control.

Please replace the paragraph beginning at line 24, Page 16 and continuing to Page 17, with the following rewritten paragraph:

Epidermal cells were counted for both the control and 3T3 frozen in triplet, and the average of the results were shown in Fig. 1 for comparison. As shown in Fig. 1, more epidermal cells were adhered and proliferated in the culture vessel according to the present invention (3T3 frozen) when compared to those grown by inoculating epidermal cells according to the conventional feed layer culture method (control), which employed 3T3 mouse ~~tung~~ embryo fibroblast as feeder cell, and incubating for 8 days.

Please replace the paragraph beginning at line 7, Page 17, with the following rewritten paragraph:

Established 3T3 mouse ~~tung~~ embryo fibroblasts were inoculated in a culture flask (culture surface: 25 cm<sup>2</sup>) at  $3 \times 10^3$  cells/cm<sup>2</sup> and incubated in a CO<sub>2</sub> incubator (at 37°C, 5% CO<sub>2</sub>) for 4 days. The medium was Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (DMEM + 10%FBS).

Please replace two paragraphs beginning at line 17, Page 17 and continuing to Page 18, with the following rewritten paragraph:

Epidermal cells collected from human skin were inoculated in the culture flask at  $1 \times 10^4$  cell/cm<sup>2</sup>. The medium was Green medium supplemented with 3% fetal bovine serum. After 4 days, the colonies of epidermal cells were observed. The epidermal cells were proliferated without contamination with 3T3 mouse ~~tung~~ embryo fibroblasts.

### EXAMPLE 3

Established 3T3 mouse ~~lung~~ embryo fibroblasts were inoculated in a culture flask (culture surface: 25 cm<sup>2</sup>) at  $3 \times 10^3$  cells/cm<sup>2</sup> and incubated in a CO<sub>2</sub> incubator (at 37°C, 5% CO<sub>2</sub>) for 4 days.

The medium was Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (DMEM + 10%FBS).

Please replace the paragraph beginning at line 27, Page 18 and continuing to Page 28, with the following rewritten paragraph:

Established 3T3 mouse ~~lung~~ embryo fibroblasts were inoculated in a culture flask (culture surface: 80 cm<sup>2</sup>) at  $3 \times 10^3$  cells/cm<sup>2</sup> and incubated in a CO<sub>2</sub> incubator (at 37°C, 5% CO<sub>2</sub>) for 3 days. The medium was Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (DMEM + 10%FBS).

### IN THE CLAIMS:

Please amend the claims as follows:

10. (Amended) The method according to Claim 1, wherein said fibroblast is 3T3 mouse ~~lung~~ embryo fibroblast.

20. (Amended) The culture vessel according to Claim 16, wherein said fibroblast derived from a mammal is 3T3 mouse ~~lung~~ embryo fibroblast.